# INVESTIGATION ON THE EFFECT OF SOPHOROLIPID AND ITS DERIVATIVE IMT-C3 ON CELL GROWTH AND MORPHOGENESIS IN *CANDIDA* SPECIES

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I, **Mansi Vij**, Roll No. **2K21/IBT/03** of M.Tech (Industrial Biotechnology), hereby declare that the Project Dissertation titled **"Investigation on the effect of sophorolipid and its derivative IMT-C3 on cell growth and morphogenesis in *Candida* species"** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in fulfilment of the requirement for the award of the Degree of Master of Technology, is original and not copied from any source without proper citation. The work has not been previously formed the basis for award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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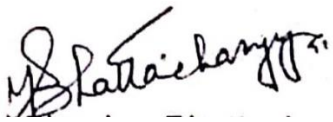

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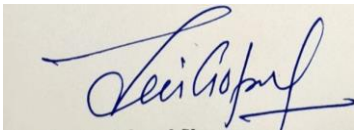

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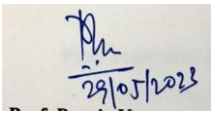


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***MANSI VIJ***

# ABSTRACT

Sophorolipid (SL) is an emerging class of biosurfactant that explicitly exhibits various applications. Amongst which the therapeutic applications are the most targeted arena for the researchers. SL being a glycolipid biosurfactant proves to be one of the most promising biosurfactant for industrial applications, along with rhamnolipids. In order to check its potency in the prevailing sectors, the antifungal activity of SL and its derivative IMT-C3 was significantly observed following a number of experiments with *Candida albicans*, along with four others non albicans *Candida* species. *Candida albicans* causes life threatening systemic infections, thereby, sometimes is difficult to treat. Thus, it remains the major causative agent for infections. Apart from this, other non albicans *Candida* species are also becoming prevalent including; *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Candida krusei*. Immunocompromised patients are highly susceptible to such infections. Various antifungal drugs are being employed to cure the infections but due to their severe toxicity and certain other limitations, there is a paradigm shift to the employment of biosurfactants. Biosurfactants show various properties such as low toxicity, reduction in surface tension, anti-microbial activities and many more. SL produced by *Starmerella bombicola* is used for the study. This naturally synthesized SL is derived in two forms. *i.e.*, acidic and lactonic form. Lactonic form of SL was used for carrying out the experiments. Due to its less toxicity, SL proves to be a potent candidate for food and pharmaceutical domains. In this study, production, synthesis, purification and characterization of SL and its derivative IMT-C3 was carried out. Antifungal effect of SL was also checked against all the 5 strains of *Candida* species.

**Keywords:** Biosurfactants, *Candida*, IMT-C3, Sophorolipid, Surfactants

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# LIST OF SYMBOLS AND ABBREVIATIONS

1. **µL:** micro-litre
2. **µg:** micro-gram
3. **mL:** milli-litre
4. **mg:** milli-gram
5. **g:** gram
6. **L:** litre
7. **nm:** nanometre
8. **hrs:** hours
9. **min:** minute
10. **rpm:** revolutions per minute
11. **β:** beta
12. **°C:** degree Celsius
13. **OD:** Optical density
14. **ND:** Not determined
15. **Amp B:** Amphotericin B
16. **SDS:** Sodium dodecyl sulfate
17. **CTAB:** Cetyl Trimethyl Ammonium Bromide
18. **SL:** Sophorolipid
19. **DMSO:** Dimethyl sulphoxide
20. **YPD:** Yeast Peptone Dextrose
21. **FBS:** Fetal Bovine Serum
22. **MIC:** Minimum Inhibitory Concentration
23. **CA:** *Candida albicans*
24. **CP:** *Candida parapsilosis*
25. **CG:** *Candida glabrata*
26. **CT:** *Candida tropicalis*
27. **CK**: *Candida krusei*
28. **SB:** *Starmerella bombicola*

# CHAPTER 1: INTRODUCTION

SURFace ACTive AgeNTS or SURFACTANTS are the generic term given to amphipathic molecules with the ability to synthesize micelles. Surfactants are effective emulsifiers, dispersing, and foaming agents due to their surface activating properties [1]. They alleviate the interfacial tension of aqueous media (such as oil-water or water-oil) or liquid-solid (including the wetting phenomena) systems, as well as the surface tension of aqueous media (such as air-water). They facilitate polar molecules to dissolve in organic solvents. Surfactants, the active constituents of soaps and detergents, are prominently incorporated to remove oil-based materials from a certain media [2][3]. Numerous industrial processes that necessitate emulsification, foaming, detergency, wetting, dispersion, or solubilization employ these materials [4]. Surfactants can be biological or synthetic in origin [3]. Synthetic surfactants are those derived from chemically based components, whilst biosurfactants are those derived from biologically based components [5][6]. Linear alkyl benzene sulphonates, α-olefin sulphonates, alcohol ether sulphonates, alcohol sulphonates, etc. are some of the prominent synthetic surfactants [3]. Biosurfactants exist in a wide range, such as glycolipids, phospholipids, lipopeptides, and fatty acids. Each type has distinct qualities that make it appropriate for exclusive reasons.

## 1.1 Biosurfactants

An organic substance known as a biosurfactant is one that is synthesized by microorganisms like bacteria, yeast, and fungi. Biosurfactants are environmentally benign since they are biodegradable as well as non-toxic, in contrast to synthetic surfactants. Biosurfactants were employed in a variety of industries, including organic chemicals, petroleum, petrochemicals, mining, metallurgy (primarily bioleaching), agrochemicals, fertilizers, foods, drinks, cosmetics, pharmaceuticals, and many others because of their special functionality. They can be employed as demulsifiers, wetting agents, foaming agents, spreading agents, functional food elements and detergents in addition to serving as emulsifiers. The potential of biosurfactants to minimise interfacial surface tension made them crucial in the bioremediation of heavy crude oil and oil recovery [7]. In addition to improving oil recovery in the petroleum business, biosurfactants can also be used as emulsifiers and stabilizers in the food industry, as well as to enhance the cleaning power of detergents and other cleaning products. Since they can solubilize and remove toxins from soil and water, they can also be employed in environmental

remediation [8]. The hydrophilic as well as hydrophobic regions that biosurfactants have cause them to congregate at the interfaces of fluids with different polarity, for instance hydrocarbons and water [9][10]. Biosurfactants have a number of benefits over synthetic surfactants, including biodegradability, biocompatibility, and digestibility. By biodegrading and detoxifying industrial effluents and bioremediating contaminated soil, the biosurfactants are capable of being used in environmental cleanup. They ended up being the most popular surfactants because to their specificity and accessibility of raw ingredients [8][11].

### 1.2 Types of biosurfactants

Chemically synthesized surfactants are typically categorized according to their polarity, while biosurfactants are typically categorized by their microbiological origin and chemical makeup. Biosurfactants are also categorized on the basis of molecular mass. *i.e.*, low molecular weight biosurfactants (includes glycolipids, lipopeptides and phospholipids) and high molecular weight biosurfactants (includes polymeric and particulate biosurfactants) [12]. The various types are enlisted below:

**1.2.1 Glycolipids:** They are long-chain aliphatic acids, hydroxyaliphatic acids attached to carbohydrates via an ester group. Majority of biosurfactants are glycolipids. The most widely recognized glycolipids are sophorolipids, trehalolipids and rhamnolipids [13].

**1.2.1.1 Sophorolipids:** These are glycolipids derived from yeasts, and have the structure of a dimeric carbohydrate called sophorose coupled by a glycosidic bond that binds a long-chain hydroxyl fatty acid [14]. Anomeric carbon of the sophorose moiety (C1′) forms the -glycosidic bond with the terminal or sub-terminal carbon of the hydroxylated fatty acids. The carboxylic groups from the fatty acids can either be free (acidic form) or internally esterified (lactonic form) [15][16].

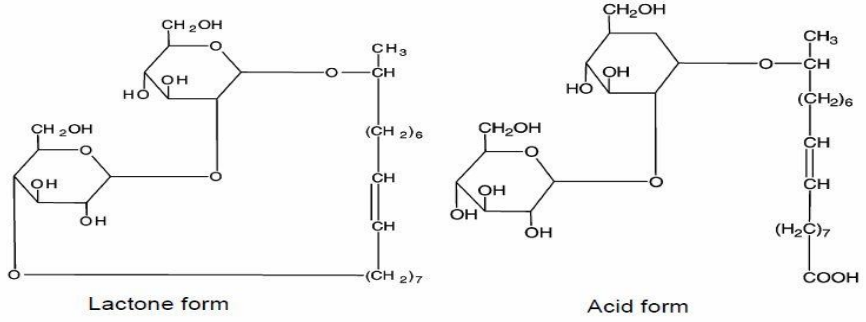



**Figure 1:** Structure of lactonic and acidic forms of sophorolipids

**1.2.1.2 Trehalolipids:** Trehalose lipids, also known as trehalolipids, are glycolipids that have a hydrophilic trehalose component. They work to reduce the surface as well as interface tension of the water [17]. These go in tandem with a vast majority of *Mycobacterium*, *Nocardia* and *Corynebacterium* species [18].

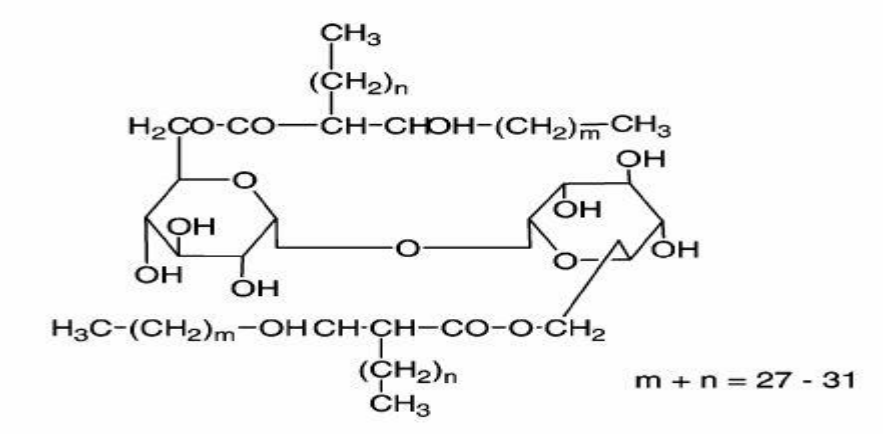



**Figure 2:** Structure of Trehalolipid

**1.2.1.3 Rhamnolipids:** Rhamnolipids are glycolipids in which one or two rhamnose molecules are joined to one or two hydroxydecanoic acid molecules. The main group of glycolipids synthesized by *Pseudomonas aeruginosa* are the highly studied biosurfactant [19].

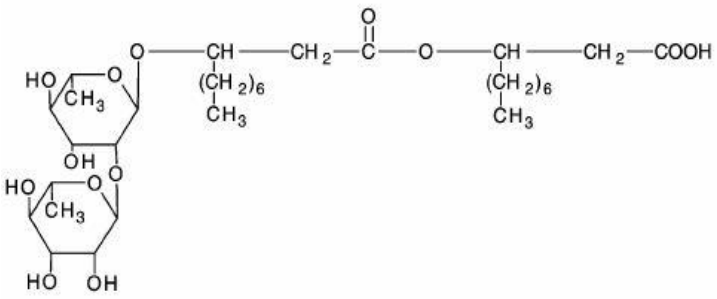


**Figure 3:** Structure of Rhamnolipid

**1.2.2 Lipopeptides:** A compound made up of a lipid and a peptide is termed as a lipopeptide. Various researchers have reported the antibacterial as well as antifungal properties of iturin, a lipopeptide, synthesized from *Bacillus subtilis* and *Bacillus licheniformis* [3][20][21]. Numerous bacterial species that produce lipopeptide biosurfactants exhibit insecticidal action against the fruit fly

*Drosophila melanogaster*, making them suitable for application as biopesticides [22]. Biosurfactants such as Cyclic Lipopeptide (CLP) retain their surface-active properties when heated at high temperatures and are stable across a wide pH range (7.0 to 12.0). Their remarkable compatibility and stability make it more advantageous to include them in the manufacturing of laundry detergents [23].

**1.2.3 Phospholipids, Fatty acids and neutral lipids:** Massive quantities of fatty acids and phospholipid surfactants are produced by many bacteria and yeasts when they thrive on n-alkanes [24]. *Acinetobacter* species produce phosphatidyl ethanolamine-rich vesicles that create optically transparent microemulsions of alkanes in water. These biosurfactants are necessary for use in therapeutic medicine [8]. Prematurely born children's respiratory failure is determined to be mostly caused by a deficit in the phospholipid protein complex. Additionally, it has been proposed that the genes that are accountable for producing such a surfactant can be isolated and cloned to be used in their fermentative synthesis [24][25].

**1.2.4 Polymeric biosurfactants:** It is widely employed as a stabilizing agent for the emulsions [3]. These include various polysaccharide-protein complexes, emulsan, liposan, alasan, mannoprotein and lipomannan; and have undergone extensive research. Even at concentrations as low as 0.001-0.01%, emulsan is a powerful emisifying agent for hydrocarbons in water [8]. *Candida lipolytica* produces the extracellular water-soluble emulsifier known as liposan, which is mostly made up of 83% carbohydrates and 17% proteins [26][27]. *Saccharomyces cerevisiae* produces mannoproteins, that consist of 17% protein and 44% mannose [4].

**1.2.5 Particulate biosurfactants:** These are the extracellular membrane vesicles. These divide to create a microemulsion, which is crucial for microbial cells to absorb alkanes [24]. Protein, phospholipids, and lipo-polysaccharide make up the vesicles of *Acinetobacter* spp. strain HO1-N, which have a diameter of 20–50 nm having a buoyant density of 1.158 cubic gcm [27][28].

### 1.3 Properties of Biosurfactants

Biosurfactants proved appropriate for commercial applications due to their special and distinctive characteristics in comparison to their chemically synthesized equivalents and the wide availability of substrates [8]. Their surface activity, resistance to pH, temperature, and ionic strength, biodegradability, low toxicological profile, emulsifying and demulsifying ability are some of the distinguishing characteristics of microbial surfactants [27]. The vast majority of biosurfactants share certain traits that make them superior to traditional surfactants, as outlined below:

**1.3.1 Surface activity:** A good surfactant must have both efficiency and efficacy. Effectiveness is determined by surface and interfacial tensions, whereas efficiency is determined by the critical micelle concentration (CMC) [29]. While the surface tension (oil/water) and interfacial (oil/water) tensions are roughly 1 and 30 mN/m, respectively, the CMC of biosurfactants varies from 1 to 2000 mg/L [24]. Surface tension and interfacial tension are both decreased by surfactant. *Bacillus subtilis* producing surfactin can lower water's surface tension to 25 mN $m^{-1}$ and the interfacial tension between water as well as hexadecane to a value less than 1 mN $m^{-1}$ [30]. The rhamnolipids produced by *Pseudomonas aeruginosa* reduced the interfacial tension between water and hexadecane to less than 1 mN $m^{-1}$ and the surface tension of the water to 26 mN $m^{-1}$ [31]. The CMC of biosurfactants is generally lower than that of chemical surfactants, *i.e.*, less surfactant is required to achieve the maximum reduction in surface tension [1].

**1.3.2 Resistance to pH, temperature and ionic strength:** The majority of biosurfactants and their surface activity are resistant to environmental parameters like pH, temperature and ionic strength [8]. High temperatures as well as pH levels between 2.0 to 12.0 facilitate the use of many biosurfactants. In contrast to synthetic surfactants, which must be inactivated by 2% NaCl, biosurfactants may tolerate salt concentrations of up to 10% [24]. It was discovered that the lichenysin from *Bacillus licheniformis* was tolerant to temperature as high as 50°C, pH between 4.5 and 9.0, and sodium chloride and calcium concentrations as high as 50 and 25 g/L, respectively [32]. A second biosurfactant, derived from *Arthrobacter protophormiae* emerged to possess both the stabilities. *i.e.*, pH (2.0–12.0) and thermostability (30–100°C) [33]. Since commercial activities are

subjected to elevated levels of pressure, pH and temperature, it is important to separate novel products from microbes that can thrive in these environments [21].

**1.3.3 Biodegradability:** When compared to synthetic surfactants, compounds derived from microorganisms can be easily destroyed and are useful for environmental applications like bioremediation/biosorption [34][35]. With a 90% clearance efficiency after 30 minutes of treatment, the biodegradable biosurfactant sophorolipid was used by researchers to inhibit the growth of the marine alga Cochlodinium [36]. As environmental concerns grow; we are forced to look for alternate products like biosurfactants [21].

**1.3.4 Low toxicological profile:** The application of biosurfactants in food, skincare products, and medications is made possible by their low level of toxicity [24]. Scientists evaluated the toxicity as well as mutagenicity profiles of *Pseudomonas aeruginosa* biosurfactants with those of chemically synthesized surfactants and concluded that the biosurfactant was neither hazardous nor mutagenic [8]. Biosurfactant, sophorolipids from *Candida bombicola* were beneficial for the food industry due to their low toxicity profile [37].

**1.3.5 Emulsifying and demulsifying ability:** Biosurfactants are capable of serving as either emulsifiers or de-emulsifiers. They have a minimal level of stability; which can be increased by additives like biosurfactants and hence can be kept as long-lasting emulsions lasting from months to years [8]. Liposan, a water-soluble emulsifier produced by the yeast *Candida lipolytica*, has been employed to coat oil droplets and emulsify edible oils, resulting in stable emulsions. These liposans were frequently utilised in the food and cosmetics sectors to develop stable oil/water emulsions [38].

## 1.4 Applications of Biosurfactants

There are numerous biotechnological uses for biosurfactants, including in the production of petroleum, food, drink, skincare products, detergents, fabrics, paints, mining, cellulose, pharmaceutics, and nanotechnology [39]. The petroleum sector is the primary market at the moment. The removal of oil residue from storage tanks, various oil recovery procedures, the cleanup of oil spills, and the bioremediation of soil as well as water all make use of biosurfactants [40][41]. The explanation of few applications is listed below:

**1.4.1 Food industry:** In addition to their prominent role as mediators that reduce surface as well as interfacial tension, hence enabling the creation and stabilisation of emulsions, surfactants can serve a variety of other purposes in the food industry [27]. For instance, to regulate the accumulation of fat globules, stabilize aerated systems, improve the consistency and shelf life of products incorporating starch, alter the rheological characteristics of wheat dough, and enhance the consistency and texture of products containing fat [42]. Through the inclusion of rhamnolipid, dough stability, volume, texture, and preservation of bread items are all improved [43]. Rhamnolipids have the potential to be used to enhance the qualities of butter cream as well as frozen confectionary items, according to the study. L-Rhamnose has a lot of potential as a flavour precursor [43][8]. Cookies, mayonnaise, ice cream, and other food products that contain water/oil emulsions are stabilized by a mannoprotein synthesized by the yeast *Saccharomyces cerevisiae*. Biosurfactants have not yet been widely utilised by the food industry [44][45].

**1.4.2 Elimination of petroleum and oil contamination:** Petroleum is a vital source of energy and the engine of economic growth [24]. The first evidence that hydrocarbon culture media encouraged the growth of a *Pseudomonas aeruginosa* strain that produced rhamnolipids was presented by Itoh and Suzuki [46]. Recent study data discoveries validated the impacts of biosurfactant on hydrocarbon biological degradation by enhancing their biodegradation by boosting microbial availability to recalcitrant substrates [47][48]. At concentrations over the Critical Micelle Concentration (CMC), biosurfactants augment the apparent solubility of certain organic compounds, increasing their accessibility for microbial absorption [49]. Researchers have found that several species of *Pseudomonas aeruginosa* as well as *Bacillus subtilis* generated rhamnolipid, a frequently isolated glycolipid;

and surfactin, a lipoprotein, respectively. These two biosurfactants have also been shown to stimulate native microorganisms for improved biodegradation of diesel-contaminated soil [50]. Due to the ease with which biosurfactants can be incorporated into a bioremediation process for a hydrocarbon-polluted environment, this approach has tremendous potential [51].

**1.4.3 Bioremediation:** By enhancing the availability of resources (such as nutrients and oxygen), environmental factors (such as pH and moisture content), and predominant microorganisms, bioremediation entails accelerating natural biodegradative mechanisms in contaminated environments. However, with the development of advanced genetic technologies, it is anticipated that the rise in bioemulsifier concentration during bioremediation will be achieved by the addition of bacteria that overproduce bioemulsifiers. Bioemulsifiers can be used as an adjuvant for accelerating the bioremediation process. The sanitation of oil pipes has lately been accomplished with success using this strategy [8]. To improve oil bioremediation, cultures of *Acinetobacter radioresistens*, which synthesize the bioemulsifier alasan but cannot use hydrocarbons as a source of carbon, were mixed to a combination of bacteria that break down oil. [52]. Biosurfactants contribute to bioremediation in at least two ways; either by expanding the surface area of hydrophobic, water-insoluble compounds or by enhancing its bioavailability of hydrophobic substances [24]. In order to reduce surface tension of a fluid by elevating its surface area and causing molecular rearrangements, hydrophobic-hydrophilic interactions are facilitated by biosurfactants. These interactions also promote bioavailability and subsequently biodegradability [53][54].

**1.4.4 Skin care arena:** Due to their skin-friendly attributes, biosurfactants have established an attractive market in the healthcare and beauty industries. As skin moisturizers, sophorolipids can be esterified or combined with ethylene oxide [3]. Biosurfactants are used in repellents for insects, acne pads, antacids, cleansing products, anti-dandruff goods, contact lens treatments, baby products, mascara, lipsticks, toothpaste, and other products as foaming agents, emulsifiers, solubilizers, wetting agents, cleansers, antimicrobial agents, and mediators of enzyme action [55]. The genus *Pseudozyma* produces mannosylerythritol lipids

(MELS), which have hair-care qualities like restoring the damaged hair. MELS are being considered as a new element for hair care products. They are a highly effective agent for both restoring damaged hair and producing smooth, flexible hair [56]. About 2% of rhamnolipid infused in water is utilized to create shampoo using rhamnolipid bio-surfactant from *Pseudomonas aeruginosa*. The antibacterial properties of the aforementioned biosurfactant kept the scalp odor-free and shiny for three days [57]. Due to their low toxic effects, biological function, biocompatibility, and biodegradability, which evidently enhance product efficiency, efficacy, and the economics, they are widely used in personal care and cosmetic goods [4].

**1.4.5 Therapeutic applications:** Due to their fungicidal, bactericidal, insecticidal, and antiviral potential as well as their use as anti-adhesive agents and enzyme inhibitors, biosurfactants have also been utilised in several biological (therapeutic) applications [58][59][60]. Many rhamnolipids have antibacterial properties. A study by Abalos et al. [61] examined the antibacterial qualities of the mixture after identifying six rhamnolipids from culture of *Pseudomonas aeruginosa* AT10 cultured on effluent from a soybean oil refinery. At doses ranging from 16 to 32 μg/mL, these rhamnolipids demonstrated good antifungal activity against several fungi [24]. Sophorolipids from *Candida bombicola* inhibited the growth of gram-negative as well as gram-positive bacteria, with minimum inhibitory concentration (MIC) of roughly 30 and 1 mg/mL in contact times of 2 and 4 hours respectively; for *Escherichia coli* (ATCC-8739) as well as *Pseudomonas aeruginosa* (ATCC-9027); and 6 and 1 mg/mL in contact times of 4 hours respectively; for *Staphylococcus aureus* (ATCC-6358) and *Bacillus subtilis* (ATCC-6633) [62]. Daptomycin is one lipopeptide that has achieved commercial antibiotic status [63]. Daptomycin, sold under the name Cubicin®, constitutes a branched cyclic lipopeptide that Cubist Pharmaceuticals develops from *Streptomyces roseosporus* cultures. Daptomycin have been further noted to exhibit potent antibacterial properties against other significant infections, including *Streptococcus pneumoniae*, which is resistant to penicillin, coagulase-negative *Staphylococci*, glycopeptide-intermediate-susceptible *Staphylococcus aureus*, and *Enterococci*, which are resistant to vancomycin [64].

# CHAPTER 2: REVIEW OF LITERATURE

## 2.1 Historical background

A form of surfactant known as a biosurfactant is created by living things like bacteria, fungi, and yeast. These natural surfactants have drawn a lot of interest because of their distinctive qualities and prospective uses across a range of sectors. With the development of synthetic surfactants in the beginning of the 20th century, the idea of surfactants first came into existence. However, studies into the synthesis and characteristics of surfactants drawn from microbes didn't start until the 1950s. The history probably dates back when a pig lung was filled with an isotonic gum solution in 1929 by Kurt von Neergaard; German-born physiologist studying in Switzerland, in an effort to "eliminate surface tension of the air tissue interfaces" [65]. He came to the conclusion that "a lower surface tension would be beneficial for the respiratory mechanism" and that "Surface tension as the force counteracting the first breath of the newly born should be investigated further" after filling the lung with air and liquid. Unfortunately, von Neergaard did not heed his own counsel, and Peter Gruenwald, a pathologist in New York, replicated these tests on the lungs from stillborn babies 18 years later [66]. The resistance to aeration, according to Gruenwald, is caused by surface tension, which opposes the admission of air. He also demonstrated how surface-active chemicals reduced the pressure required for aeration [65].

Ellen Avery was a key figure in figuring out how respiratory distress syndrome (RDS) develops. Prior to that, John Clements from the US Army Chemical Centre in Maryland, USA; Charles Macklin in Canada, and Richard Pattle in England investigated the impact of nerve gases on the lungs in the early 1950s. Each made a significant contribution to understanding the significance of surfactant in their own unique way [65][67][68]. Marshall Klaus and his San Francisco colleagues' development work on surfactant phospholipids made it possible for the first trials using synthetic surfactants to be carried out in Canada as well as Singapore, following publications in 1964 and 1967 [69][70]. A significant observation was made in New Zealand and reported in 1969, some two years later [71]. Scientists also demonstrated in 1973 that pharyngeal deposition of natural surfactant, as opposed to tracheal instillation, was successful in lowering death from RDS, but more than 40 years later, this form of surfactant administration is still not widely accepted [72]. Bengt Robertson, who is skilled in testing surfactants in vitro and in vivo, and Tore Curstedt, a clinical chemist with an interest in protein

and phospholipid isolation, teamed together in Stockholm [73]. Together, they created a porcine surfactant that they collectively dubbed Curstedt-Robertson surfactant, or simply Curosurf. In addition to being made from pig lungs rather than cow lungs, this surfactant was distinctive in that it underwent a further preparation phase of liquid gel chromatography, which removed everything but polar lipids, SP-B and SP-C, which had a phospholipid content of 80 mg/mL [65][73]. Comparison of synthetic surfactant Turfsurf versus Curosurf in vitro and using Bengt's 27-day fetal rabbit model was carried out in Stockholm in November 1984. The varied chamber, persistent pressure whole body plethysmograph, created by Burkhard Lachmann and Bengt Robertson was used to ventilate the 27-day fetal rabbits while administering surfactant or saline which was subsequently improved by Bo Sun. In treating experimental RDS, Curosurf was unquestionably superior to Turfsurf [74].

Numerous surfactant preparations, including the initially developed protein-free synthetic surfactants pumactant (ALEC), colfosceril palmitate (Exosurf), and the Belfast surfactant (Turfsurf), which was never commercially produced, have been used in clinical trials. Natural (minced lung extract) surfactants include Surfactant-TA (Fujiwara's surfactant), beractant (Survanta), which is the North American version of Sur Allen Merritt along with Mikko Hallman conducted very intriguing research on the amniotic fluid extract, which contained considerable SP-A in along with SP-B and SP-C and seemed to be quite beneficial [65][75]. When the potential of HIV transmission became clear, it was taken out of clinical use. Recent research has focused on a few fresh synthetic surfactants that incorporate protein counterparts, such CHF5633 [73]. Beractant, Bovactant, and Poractant Alfa are the three surfactant preparations that are now approved for usage in Europe, whereas Beractant, Calfactant, Lucinactant, and Poractant Alfa are the four. The exception is lucinactant, which comes from animal lungs. Beractant, Bovactant, and Poractant Alfa are the three surfactant preparations that are now approved for usage in Europe, whereas Beractant, Calfactant, Lucinactant, and Poractant Alfa are the four approved in USA. The exception is lucinactant, which comes from animal lungs [76]. Surfactant being the first medication created specifically for the treatment of newborns, marking a significant advance in neonatal care over the previous 35 years. Surfactant decreased pulmonary air leakage and infant mortality by around 50%. Additionally, the adoption of it led to a 6% decrease in newborn mortality in United States of America [65].

## 2.2 Emergence of biosurfactants

Due to its novel structural design, adaptability, and variety of capabilities, biosurfactants have lately gained recognition as intriguing molecules with promise for usage in a wide range of therapeutic applications. These molecules can interact with the cell membranes of various animals and/or the environment primarily as a result of their surface activity, and can therefore be considered as potential cancer therapies or as components of systems for the delivery of drugs. It has been demonstrated that some forms of microbial surfactants, including lipopeptides and glycolipids, preferentially limit the growth of cancer cells and rupture cell membranes, leading to the lysis of the cells through apoptotic pathways. Additionally, applications for using biosurfactants as medicine delivery systems are both financially and scientifically intriguing [77]. Biosurfactants are a broad class of amphipathic molecules with unique chemical structures that are made by a variety of microorganisms. These compounds, which are mostly generated as the secondary metabolites, play important roles in the survival of the microorganisms that produce them by facilitating the movement of nutrients, interfering with interactions between microbes and their hosts and quorum sensing systems, or acting as biocide agents [78].

Numerous investigations on their potential therapeutic applications have been spurred by their biological makeup and recognized promise. These substances have advantages over synthetic surfactants since they are derived from microorganisms, are biodegradable, and have minimal toxicity. Due to this, they have received extensive research for bioremediation, increased oil recovery, and applications in the food and cosmetics industries [78][79][80]. They are typically categorized as low and high molecular weight biosurfactants, which include polysaccharides, proteins, lipoproteins, and glycolipids and lipopeptides, respectively. Due to their generally simpler structures, low molecular weight biosurfactants typically have great surface-active characteristics. Surfactin (lipopeptide) and rhamnolipids (glycolipids) are two of the finest researched biosurfactants [77]. Since they partition at the boundaries of fluid phase with different polarity and hydrogen bonds, biosurfactants have the ability to influence the adherence of microorganisms [80][81][82]. Additionally, it has been found that microbial biosurfactants outperform plant-based surfactants in terms of scale-up capability, manufacturing speed, and multifunctionality. Many plant-based biosurfactants, such as saponins, lecithins, and soy proteins, have good emulsifying capabilities but are costly to produce on an industrial scale and have other questionable qualities including solubility and hydrophobicity [83].

Rhamnolipids, a group of glycolipid biosurfactants, were identified from the bacterium *Pseudomonas aeruginosa* in the 1960s by German researchers. Rhamnolipids were discovered to be effective emulsifiers and surface-active substances [84]. The "rhamnolipids" category of glycolipid biosurfactants stands out among the other biosurfactant subcategories. A rhamnose sugar molecule is joined by the carboxyl end of a β-hydroxy fatty acid to form rhamnolipid, which is largely a crystalline acid. Mono- and di-rhamnolipids are two types of rhamnolipids that *Pseudomonas aeruginosa* mostly produces. The Pseudomonas species *Pseudomonas chlororaphis*, *Pseudomonas plantarii*, *Pseudomonas putida* and *Pseudomonas fluorescens* are also known to synthesize rhamnolipids [85]. Kaeppeli and Guerra-Santos submitted the first patent application for the manufacture of rhamnolipids in 1984 (US 4628030), and they were successful in getting it in 1986 for their research on Pseudomonas aeruginosa DSM 2659 [86]. Wagner then applied for and was granted a patent (US 4814272) in 1989 for the biotechnological manufacture of rhamnolipids using Pseudomonas sp. DSM 2874 [87].

The antibacterial, antifungal, and antiviral actions of biosurfactants, along with their anti-adhesive tendency towards pathogens and probiotic nature, are the ones that are most pertinent for applications in the medical field [77]. Certain biosurfactants have been suggested as viable substitutes for synthetic medications and antimicrobials and could be employed as therapeutic agents that are both safe and efficient. Their potential uses range from gene transfection to antigen adjuvants, fibrin clot inhibitors and activators, as well as antiadhesive coatings for biomaterials, incorporation into probiotic preparations to treat pulmonary immunotherapy and urogenital tract infections [39]. Biosurfactants have recently been demonstrated to have an impact on cancer cells. For instance, it has been discovered that the lipopeptide surfactin causes the death of breast cancer cells [88]. Similar to this, the glycolipids succinoyl trehalose lipids (STLs) and mannosylerythritol lipids (MELs) have been linked to tumour cell apoptosis and growth arrest [89][90][91]. Numerous more therapeutic uses for biosurfactants have been proposed, primarily based on their adaptable self-assembly in nanotechnology [92]. For instance, a DNA complexed with biosurfactant and β-sitosterol β-D-glucoside in a liposome vector thymidine kinase gene therapy for the herpes simplex virus was effective [93]. More recently, gene transfection efficiency has been improved in vitro as well as in vivo by using nanovectors that contain a biosurfactant [94].

The prospects for sustainable applications of biosurfactants, which have a long history, make them an intriguing topic for research and development. We may anticipate more developments and a wider application of such natural surfactants in a variety of industries as our

comprehension of biosurfactants expands. Although it is apparent that biosurfactants are worthwhile, versatile, and helpful molecules for therapeutic uses, some of them may pose a risk to humans and should be closely examined.

### 2.3 Sources of biosurfactants

It is discovered that many of the bacteria that produce biosurfactants are hydrocarbon degraders [7][95]. However, numerous studies over the past few decades have demonstrated the benefits of microbially generated surfactants for both bioremediation and increased oil recovery [7][96]. Table 1 is a list of the biosurfactants derived from microbial sources.

**Table 1.** Biosurfactants derived from various species of bacteria and fungi [8]

| Organisms | Biosurfactants |
|---|---|
| **Bacteria** | |
| *Serratia marcescens* | Serrawettin |
| *Rhodotorula glutinis, R. graminis* | Polyol lipids |
| *Rhodococcus erythropolis, Arthrobacter spp. Nocardia erythropolis, Corneybacterium spp., Mycobacterium spp.* | Trehalose lipids |
| *Pseudomonas spp., Thiobacillus thiooxidans, Agrobacterium spp.* | Ornithine lipids |
| *Pseudomonas fluorescens, Leuconostoc mesenteriods* | Viscosin |
| *Pseudomonas aeruginosa, P. chlororaphis, Serratia rubidea* | Rhamnolipids |
| *P. fluorescens, Debaryomyces polmorphus* | Carbohydrate lipid |
| *P. aeruginosa* | Protein PA |
| *Lactobacillus fermentum* | Diglycosyl diglycerides |
| **Fungi** | |
| *Torulopsis bombicola* | Sophorose lipid |
| *Candida bombicola* | Sophorolipids |
| *Candida lipolytica* | Protein-lipidpolysaccharide complex |
| *Candida lipolytica* | Protein-lipidcarbohydrate complex |
| *Candida ishiwadae* | Glycolipid |
| *Candida batistae* | Sophorolipid |
| *Aspergillus ustus* | Glycolipoprotein |
| *Trichosporon ashii* | Sophorolipid |

**2.3.1 Bacterial Biosurfactants:** Numerous organic substances are used by microorganisms as sources of energy and carbon for development. Microorganisms produce a variety of chemicals, known as biosurfactants, to enable their diffusion into the cell when the carbon source is in a form that is insoluble like a hydrocarbon [8]. Ionic surfactants that some bacteria and yeasts emit emulsify the $C_xH_y$ material in the growing medium. Rhamnolipids, which are produced by various *Pseudomonas* species [97][98], and sophorolipids, which are produced by various *Torulopsis* species, are a few of examples of this class of biosurfactant [26][99]. Other bacteria have the capacity to alter the composition of their cell walls through the production of nonionic or lipopolysaccharide surfactants. *Rhodococcus erythropolis*, varying *Mycobacterium* spp., and *Arthrobacter* spp., which create nonionic trehalose corynomycolates, are a few instances of this group [100][101]. *Acinetobacter* species create lipopolysaccharides like emulsan, and *Bacillus subtilis* produces lipoproteins like surfactin and subtilisin [30][101].

**2.3.2 Fungal Biosurfactants:** The synthesis of biosurfactants via bacterial species has received much study, but comparatively few fungi are known to do so. The ones that have been studied among fungi are *Candida bombicola* [102], *Candida lipolytica* [103], *Candida ishiwadae* [104], *Candida batistae* [105], *Aspergillus ustus* [106], and *Trichosporon ashii* [107]. Many of these have a reputation for producing biosurfactant using inexpensive basic resources. Sophorolipids (glycolipids) are the primary type of biosurfactants synthesized by these fungi. When growing on n-alkanes, *Candida lipolytica* makes cell wall-bound lipopolysaccharides [108].

## 2.4 Potent biosurfactants

Surface-active substances known as biosurfactants are created by microorganisms. They have drawn a lot of interest because of their potential uses across numerous industries. Here are some illustrations of strong biosurfactants and their advantages:

**2.4.1 Rhamnolipids:** Many bacterial species, including *Pseudomonas aeruginosa*, manufacture rhamnolipids. They have exceptional wetting, foaming, and emulsifying abilities. Among the advantages of rhamnolipids are:

i.) **Improved oil recovery:** Rhamnolipids are useful in the petroleum sector because they can improve the effectiveness of oil extraction from reservoirs.

ii.) **Bioremediation:** Rhamnolipids help hydrocarbons and heavy metals break down, making it easier to clean up contaminated locations.

iii.) **Personal care items:** In recognition of their mildness, skin-friendliness, and foamy qualities, rhamnolipids are utilised in cosmetics and shampoos [85].

**2.4.2 Sophorolipids:** Yeast species like *Candida bombicola* and *Starmerella bombicola* synthesize sophorolipids. Excellent emulsification, antibacterial, and anti-adhesive qualities are displayed by them. Among sophorolipids advantages are:

i.) **Green detergents:** Sophorolipids can be used in ecologically sustainable detergents because they are non-toxic and biodegradable.

ii.) **Improved oil recovery:** Sophorolipids, like rhamnolipids, can improve oil recovery from reservoirs.

iii.) **Applications in agriculture:** Sophorolipids have demonstrated promise as adjuvants in agricultural formulations and as biocontrol agents for use against plant diseases [24].

**2.4.3 Surfactin:** Bacillus species, like Bacillus subtilis, create surfactin, a cyclic lipopeptide biosurfactant. Exceptional surface tension lowering, foaming, and antibacterial characteristics are all displayed by it. Surfactant advantages include:

i.) **Bioremediation:** Surfactin helps hydrophobic contaminants become soluble and degrade, aiding in the cleaning up of polluted areas.

ii.) **Cosmetics:** Because Surfactin may stabilize emulsions and increase the entry of active chemicals into the skin, it is utilised in formulations for skincare and cosmetic products.

iii.) **Food preservation:** Surfactin is a natural food preservation agent plus emulsifier that can be used in the food industry to increase the shelf life of food items [29].

Glycolipid-type biosurfactants known as sophorolipids are synthesized by particular non-pathogenic yeasts (for instance, *Candida glabrata*). Sophorolipids have demonstrated various unique features, including detergent activity (a reduction in surface and interfacial tension), which is frequently used for biomedical applications. They have also demonstrated antiviral action against enveloped viruses, immunomodulation, as well as anticancer activity [109]. They are an appealing substitute for traditional surfactants in many industries due to their eco-

friendliness, variety of applications, and antibacterial activity. It is anticipated that ongoing research and development activities would increase their manufacturing efficiency and widen their scope of uses.

# CHAPTER 3: AIMS AND OBJECTIVES

The biomolecules known as biosurfactants are created by a variety of microbes. These substances have both hydrophilic as well as hydrophobic moieties, making them amphiphilic. They lessen the medium's surface tension after application. This can be an excellent source for a number of uses. The following goals were taken into account when we conducted the study.

**3.1** Production and synthesis of SL and its derivative IMT-C3

**3.2** Purification and characterization of SL and its derivative IMT-C3

**3.3** Antifungal effect of partially purified SL and IMT-C3 against *Candida* species

# CHAPTER 4: MATERIALS AND METHODS

## 4.1 Material used:

I. **HIMEDIA:** Yeast Extract Powder, Malt Extract Powder, Peptone, Dextrose/Glucose, Agar Powder, Urea, FBS
II. **SIGMA-ALDRICH:** Triton X-100, Amp B
III. **MERCK:** SDS
IV. **SPECTROCHEM:** CTAB
V. **RANKEM:** DMSO, Hexane, Ethyl acetate, Chloroform
VI. **SRL:** Silica Gel 60-120 mesh for column chromatography, Methanol
VII. **GENAXY:** 96-well plate
VIII. Cotton seed oil for sophorolipid production

**4.2 Cultures used:** *Candida albicans, Candida parapsilosis* (MTCC-6510), *Candida tropicalis* (MTCC-184), *Candida glabrata* (MTCC-3019), *Candida krusei* (MTCC-9215), *Starmerella bombicola* (MTCC-1910)

**4.3 Culture growth media used:** YPD media

**4.4 Softwares used for analysis:**

I. KC Junior for MIC analysis
II. GENE Sys for spot dilution assay analysis
III. Gen5 for time kill kinetics analysis

## 4.5 Methods:

### 4.5.1 Production and synthesis of SL and its derivative IMT-C3

In-flask production of SL was carried out which was further followed by triple ethyl acetate extraction method for the extraction of crude SL. As a result of its chemical and biological properties, such as medium polarity and low cell toxicity, solvents such ethyl acetate are employed in extraction process. The biphasic effects of ethyl acetate allow for the extraction of both polar and non-polar molecules.

**4.5.1.1 Production of SL:** For the production of SL, the seed culture of *S. bombicola* was prepared in 2 L Erlenmeyer flask containing 20 mL YPD media according to the composition of yeast extract (10 g/L), peptone (20 g/L) and dextrose/glucose (20 g/L). For the production of inoculum, a loop full of culture of *S. bombicola* cells were inoculated in the seed culture medium and was grown for 24 hours. The medium used for the production of SL contained glucose (100 g/L), malt extract (10 g/L), urea (1 g/L) and cotton seed oil (100 g/L). The pH was set at 6.5 before adding cotton seed oil to the medium. For the production of SL in the flasks, 2% of the 24-hour grown inoculum was inoculated in a 500 mL flask containing 100 mL of media. The flask was incubated for 7 days in shaking conditions of 200 rpm at 30°C.

**4.5.1.2 Extraction of SL:** After 7 days of incubation at 30°C and 200 rpm, a sticky layer of compound can be clearly visible on the sides of the flask. For the extraction, the cells were pelleted down and SL was extracted using triple ethyl acetate extraction method. The compound was then obtained in the RBF and is further subjected for the purification in order to remove impurities.

**4.5.1.3 Synthesis of IMT-C3:** For the production of the derivative of SL, designated as for now as IMT-C3 was carried out by the enzymatic synthesis of SL.

### 4.5.2 Purification and characterization of SL and its derivative IMT-C3

For the purification of crude SL obtained via ethyl acetate extraction, column chromatography was done. Column chromatography's fundamental working principle is the adsorption of the solution's solutes onto a stationary phase (silica mesh in our case), which then separates the mixture into individual components. The eluents were then collected and further checked for antifungal activity via well diffusion method on the seed plate of *Candida albicans* on YPD agar plate. In the process, the eluents when poured into the wells punctured on the agar plate diffuses into the agar and inhibits the growth of test microorganism. The diameter around the inhibition growth zones is then checked for active eluents. The biosurfactant activity was further checked via oil displacement method which is based on the fact that when biosurfactant is added onto the surface of oil, a separate zone is formed on the surface of the oil which supports one of the characteristic features of biosurfactants. *i.e.*, reduction in surface tension.

**4.5.2.1 Purification of SL:** Silica Column chromatography was performed to carry out the purification of SL. Hexane (500 mL) was added to 150 mg of silica mesh (60-120) in order to form a slurry. The slurry was sonicated for 10 minutes in order to avoid the formation of bubbles. The slurry was added to the glass column in order to pack the column with silica to form silica/matrix bed. The hexane was allowed to pass through the column. The silica bed was further equilibrated using 1% methanol having volume of 200 mL (2 mL methane + 198 mL chloroform). Further 2 g of the compound and 14 g of silica was dissolved in methanol for adsorption. The methanol was subjected to evaporation at 40°C. The fully dried silica adsorbed compound was added to the column above the silica bed to develop compound bed. Above compound bed, a cotton bed is developed in order to avoid the disturbances caused when the gradients were added for elution. Different gradients ranging from 1% to 10% having volume of 250 mL were prepared. The gradients were subsequently eluted at a constant flow rate of 1 mL/min. The eluents were labelled respectively and were subjected to rota evaporation at 40°C. The film obtained on the interior of the RBF was dissolved in 5 mL methanol. The extractions were further used to check the antifungal activity of SL for other experiments.

**Table 2.** Volume of gradients for elution

| Gradients (%) | Methanol (mL) | Chloroform (mL) |
|---|---|---|
| 1 | 2.5 | 247.5 |
| 2 | 5 | 245 |
| 3 | 7.5 | 242.5 |
| 4 | 10 | 240 |
| 5 | 12.5 | 237.5 |
| 6 | 15 | 235 |
| 7 | 17.5 | 232.5 |
| 8 | 20 | 230 |
| 9 | 22.5 | 227.5 |
| 10 | 25 | 225 |

**4.5.2.2 Antifungal activity via well diffusion method:** For checking the antifungal activity, a seed plate of *Candida albicans* was prepared in 25 mL of YPD Agar. Eleven wells were punctured in the plate (10 for eluted gradients and 1 for methanol). 50 µL of each was added in the respective labelled wells. The plate was incubated overnight at 30°C. The plates were checked the next day for the zone of inhibition depicting the gradients showing activity. The active eluents were then subjected to SpeedVac promoting solvent evaporation, thereby, leaving powdered SL behind.

**4.5.2.3 Biosurfactant activity:** In a petridish, 5 mL of oil was taken and to which different concentrations of SL were added. On application of higher concentrations of SL, a clear separate zone was formed supporting the fact that with increasing concentrations of SL, surface tension decreases.

### 4.5.3 Antifungal effect of partially purified SL and IMT-C3 on *Candida* species

For determining the antifungal effect on different strains of *Candida* species, MIC was done initially followed by spot dilution assay and Time Kill Kinetics. MIC was carried out to determine the minimum cytotoxicity concentration of a given drug or compound on the target cell. Spot dilution assay was used to determine the intensity of viable cells with various concentrations of drugs. Time Kill Kinetics method was used in order to check the activity of drugs against different strains of *Candida* species in order to determine its activity over fixed intervals of time.

**4.5.3.1 Minimum Inhibitory Concentration (MIC):** The MIC of purified SL and IMT-C3 with respect to other surfactants was determined against both *Candida albicans* as well as non albicans *Candida* species, namely; *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Candida krusei*. The MIC was carried out in YPD broth medium against the prepared stocks of 6 drugs/surfactants, namely; IMT-C3 (2X), SL (40 mg/mL), SDS (100 mg/mL), Triton X-100 (100 mg/mL), CTAB (20 mg/mL), Amp B (1 mg/mL). 10 µL of each surfactant was added to the higher concentration wells in the 96-well plate. 10 µL of YPD medium was added throughout the plate. Double dilution was then carried out from higher to lower concentration. The volume was then made upto 100 µL by adding remaining 90 µL of YPD medium to the plate. At last, 100 µL cell suspension, made from 200 µL of 0.001 OD in 10 mL YPD medium, and added to each well. Hence, making the volume of 200 µL of the entire plate. The plate was then incubated overnight at 30°C. The plate OD was determined the next day at 600 nm using the software "KC Junior" and hence MIC was calculated.

**4.5.3.2 Spot Dilution Assay:** Nine YPD agar plates were made containing 1 mL of the respective surfactants in each plate. The surfactants used were DMSO, Amp B (0.5 µg/mL), SDS (160 µg/mL), Triton X-100 (320 µg/mL), CTAB (1 µg/mL), IMT-C3 (2 µg/mL), IMT-C3 (6 µg/mL), SL (1 mg/mL), SL (2 mg/mL). Volume 200 µL of 1 OD of all the 5 species was added to higher concentration well in 96-well plate. 160 µL of YPD medium was added in rest of the 5 wells. Five-fold dilution was carried out till $6^{th}$ well. About 4 µL of the respective culture was used for spotting

on the respective drug plates. The plates were incubated for 24-48 hours and the results were then analyzed using the software "GENE Sys".

**4.5.3.3 Time Kill Kinetics:** The seed culture of all the 5 strains of *Candida* were prepared in 2 mL of YPD medium broth. Respective volume of each drug, not exceeding the maximum volume of 5 µL, was added in triplicates in each well in 96-well plate. The experiment was carried out in YPD medium broth against 6 drugs, namely; DMSO, Amp B, SDS, CTAB, IMT-C3 and SL. The final concentration of each drug varied with respect to the strain used. At last, final cell density of 0.0001 OD was maintained in each well containing 100 µL of volume.

**Table 3.** List of drugs and their test concentrations with respect to each strain of *Candida* as determined by MIC

| Drugs | CA (µg/mL) | CP (µg/mL) | CG (µg/mL) | CT (µg/mL) | CK (µg/mL) |
|---|---|---|---|---|---|
| DMSO | Control | Control | Control | Control | Control |
| Amp B | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| SDS | 100 | 625 | 320 | 625 | 625 |
| CTAB | 1 | 0.5 | 1 | 1 | 0.5 |
| IMT-C3 | 1 | 1 | 1 | 1 | 0.5 |
| IMT-C3 | 2.5 | 2.5 | 2.5 | 2.5 | 1 |
| IMT-C3 | 5 | 5 | 5 | 5 | 2.5 |
| IMT-C3 | 10 | 10 | 10 | 10 | 5 |
| IMT-C3 | - | 12.5 | 12.5 | 12.5 | 10 |
| SL | 50 | 1000 | 1000 | 1000 | 1000 |
| SL | 100 | 2000 | 2000 | 2000 | 2000 |
| SL | 200 | 3000 | 3000 | 3000 | 3000 |

# CHAPTER 5: RESULTS

## 5.1 Production and synthesis of SL and its derivative IMT-C3

**5.1.1 Production of SL:** In flask production of SL carried out by *S. bombicola* in 2 L Erlenmeyer flask upto 7 days at 200 rpm and 30°C.

**Figure 4:** In-flask production of SL by *S. bombicola*

**5.1.2 Extraction of SL:** Crude SL was extracted using triple ethyl acetate extraction and viscous dark brown crude compound was obtained.

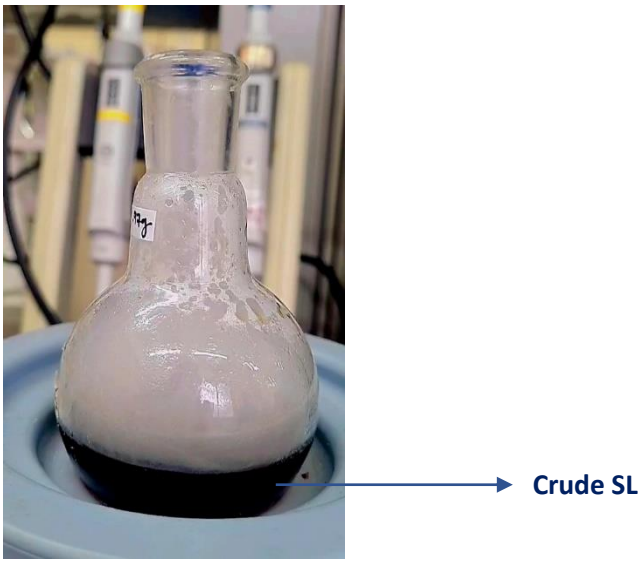



**Figure 5:** Extracted crude SL

## 5.2 Purification and characterization of SL and its derivative IMT-C3

**5.2.1 Purification of crude SL:** Crude SL was further purified using silica column chromatography technique. The gradients (1%-10%) were passed through silica column containing cotton bed followed by compound bed (crude SL adsorbed on silica) and silica/matrix bed at a constant flow rate of 1 mL/min.

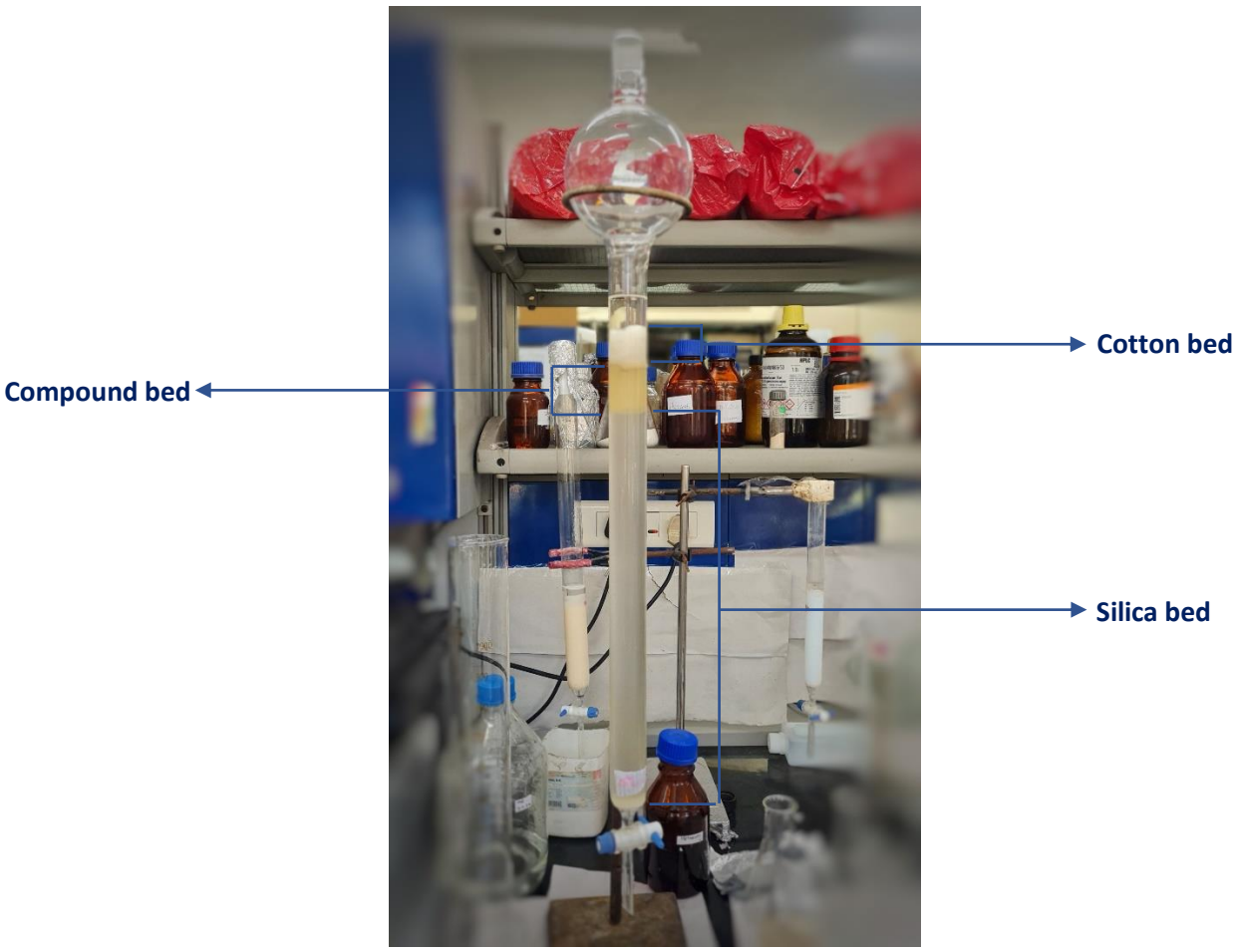



**Figure 6:** Column chromatography for purification of crude SL

**5.2.2 Antifungal activity via well diffusion method:** The eluents obtained from the column chromatography were checked further for antifungal activity against *Candida albicans*. The seed plate was incubated overnight at 30°C, we observed zone of inhibition against *Candida albicans* in eluents 1, 2, 5, 6, 7 and 8 as shown by red circle in Figure 7. Out of which, fractions 1 and 2 were less active in comparison to 5-8 eluents, which indicates early elution of crude compound in 1 and 2. While, fractions 5-8 (proceeded

for further experiments) were more active against *Candida* indicating the presence of purified compound.

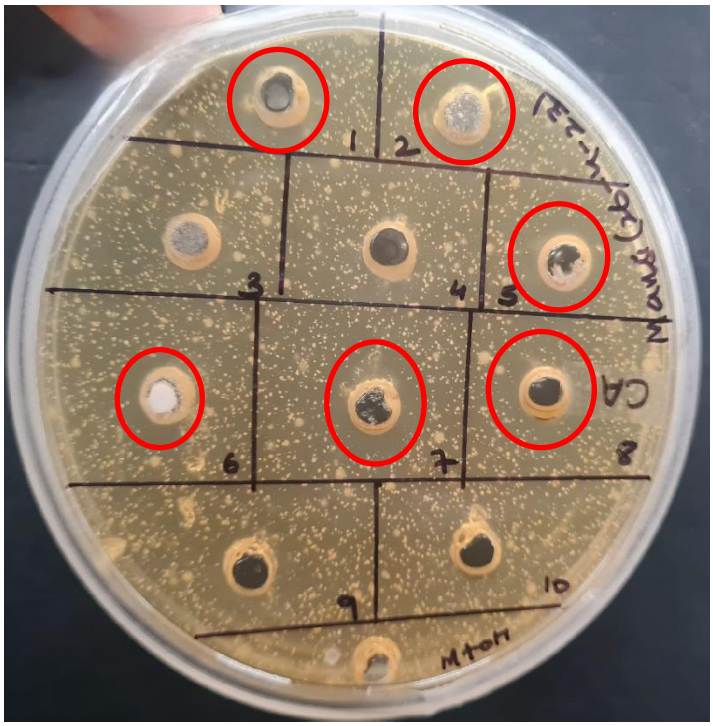
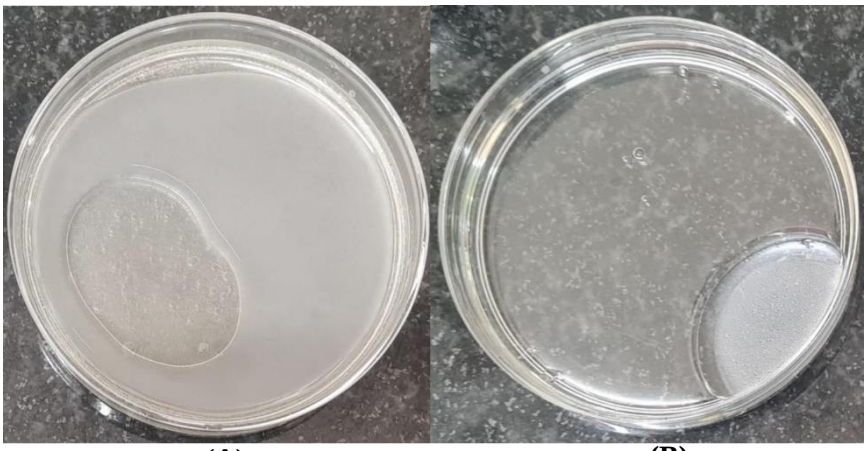



**Figure 7:** Zone of inhibition exhibiting active eluents against *Candida albicans*

**5.2.3 Biosurfactant activity:** The extracted SL was characterized for its biosurfactant activity by performing oil displacement method. At lower concentration of SL (1 mg/mL), oil displacement was not much evident as shown in Figure 8 (A), whereas, at higher concentration of SL (2 mg/mL) there was a clear displacement on the surface of oil depicting the reduction in surface tension as shown in Figure 8 (B).



**(A)** **(B)**

**Figure 8:** Surface tension reduction by oil displacement method **(A)** Effect of low concentration of SL on the oil **(B)** Effect of high concentration of SL on the

## 5.3 Antifungal effect of partially purified SL and IMT-C3 on *Candida* species

**5.3.1 Minimum Inhibitory Concentration (MIC):** The MIC of purified SL and its derivative IMT-C3 with respect to other surfactants was carried out in 96-well plate which was incubated overnight at 30°C. On the basis of observation, various MIC concentrations were determined for each drug against all the five species of *Candida*. *i.e.*, *Candida albicans* (CA), *Candida glabrata* (CG), *Candida krusei* (CK), *Candida parapsilosis* (CP) and *Candida tropicalis* (CT). Figure 9 depicts the graphical representation of cell viability in presence of SL (Figure 9A) and IMT-C3 (Figure 9B), as determined by double dilution method. Table 4 enlists the $MIC_{80}$ concentrations of various drugs as determined by the above experiment.

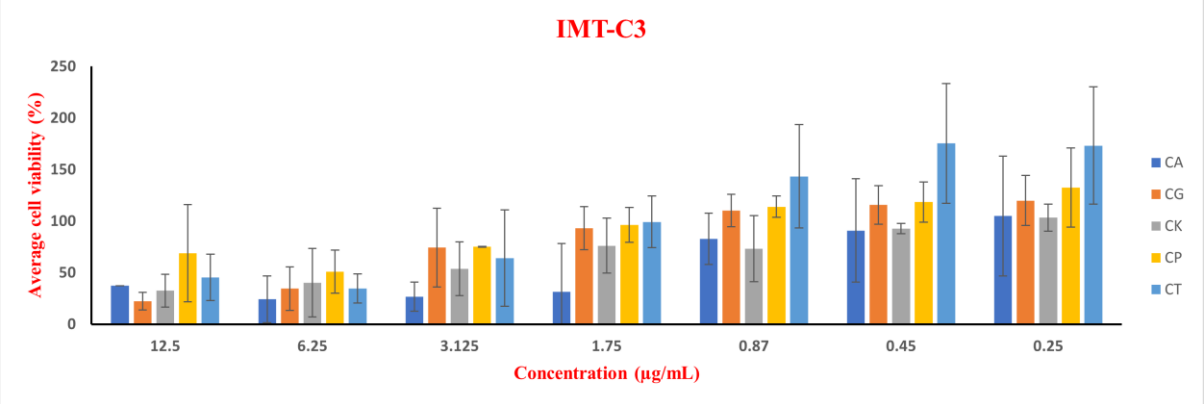



**Figure 9 (A):** Minimum Inhibitory Concentration (MIC) of IMT-C3 against various *Candida* species as determined by double drug dilution method

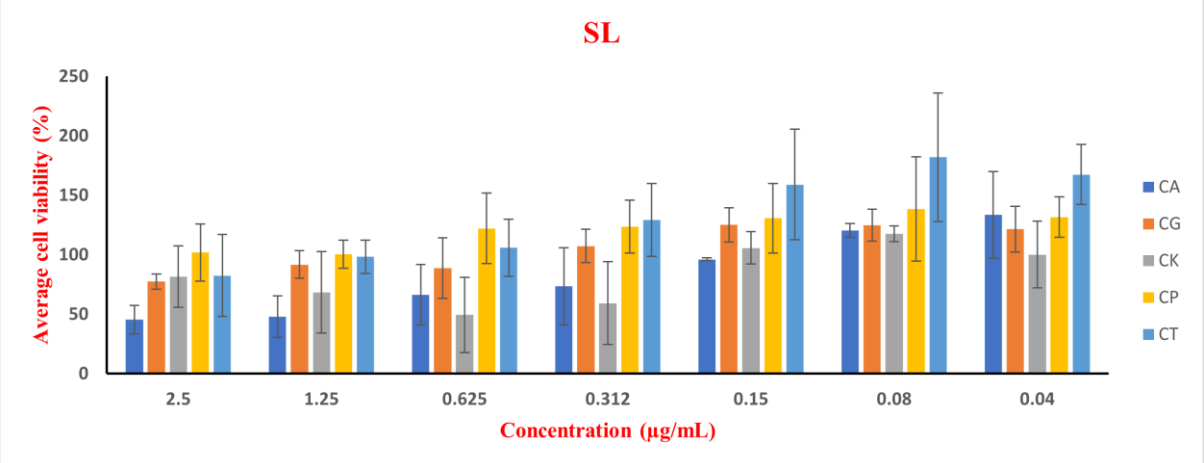



**Figure 9 (B):** Minimum Inhibitory Concentration (MIC) of SL against various *Candida* species as determined by double drug dilution method

**Table 4.** MIC of various drugs against *Candida* species as determined by double drug dilution method

| Drugs | CA (µg/mL) | CG (µg/mL) | CK (µg/mL) | CP (µg/mL) | CT (µg/mL) |
|---|---|---|---|---|---|
| C3 | 1.75 ± 16.47 | 6.25 ± 21.03 | 3.125 ± 21.68 | 6.25 ± 21.90 | 6.25 ± 39.10 |
| SL | 1250 ± 18.68 | ND | ND | ND | ND |
| SDS | 156.25 ± 12.25 | 312.5 ± 10.81 | 625 ± 16.09 | 625 ± 24.94 | 625 ± 22.74 |
| Triton | ND | ND | ND | 312.5 ± 21.05 | ND |
| CTAB | 0.78125 ± 13.68 | 0.78125 ± 1.89 | 0.390625 ± 6.66 | 0.390625 ± 22.45 | 0.78125 ± 8.37 |
| Amp B | 0.390625 ± 8.32 | 0.390625 ± 4.62 | 0.390625 ± 6.50 | 0.390625 ± 39.27 | 0.390625 ± 6.61 |

**(*Note:* CA- *Candida albicans*, CG- *Candida glabrata*, CK- *Candida krusei*, CP- *Candida parapsilosis*, CT- *Candida tropicalis*)**

**5.3.2 Spot dilution assay:** To check the viability of the *Candida* strains on solid agar medium, spot dilution assay was performed with different concentration of drugs against different strains of *Candida*.

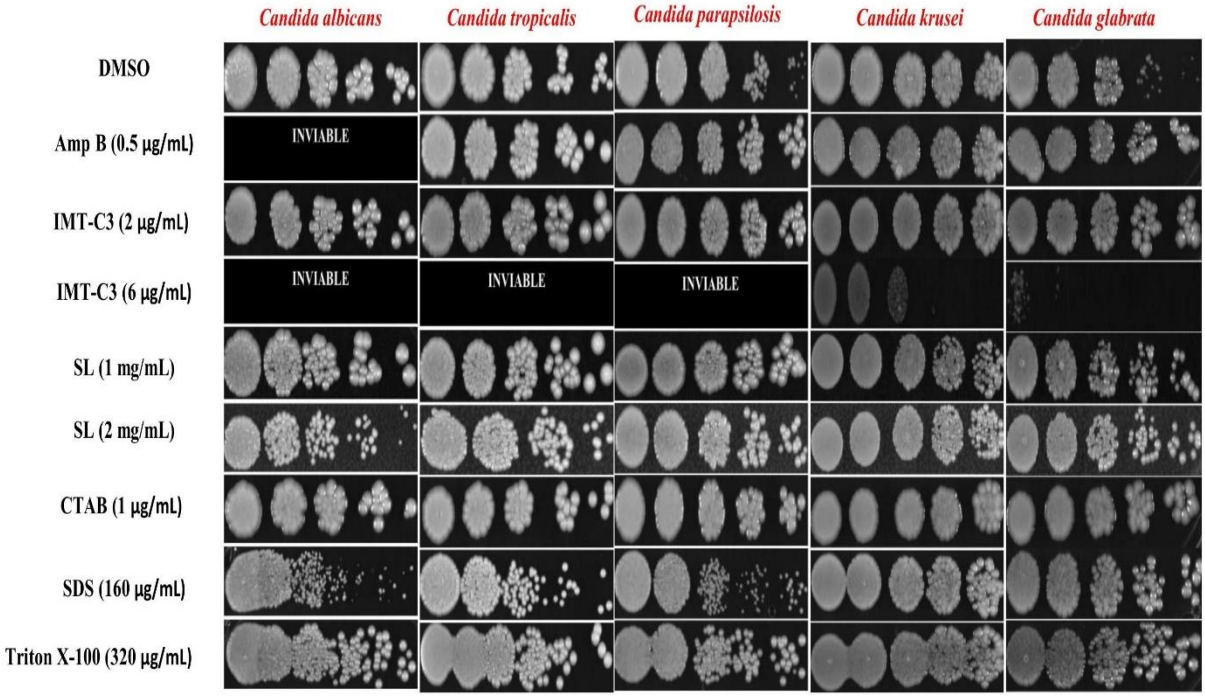



**Figure 10:** Spot assay analysis exhibiting the viability of *Candida* species with respect to different concentrations of drugs

**5.3.3 Time Kill Kinetics:** The growth kinetics of different strains of *Candida* was carried out in presence of different drugs for upto 48 hours of incubation at 30°C, $O.D_{600}$ was measured at a regular interval of 6 hours. SL exhibited a fungistatic effect with increasing concentration of drugs, whereas, IMT-C3 exhibited a fungicidal effect above the determined MIC concentrations.

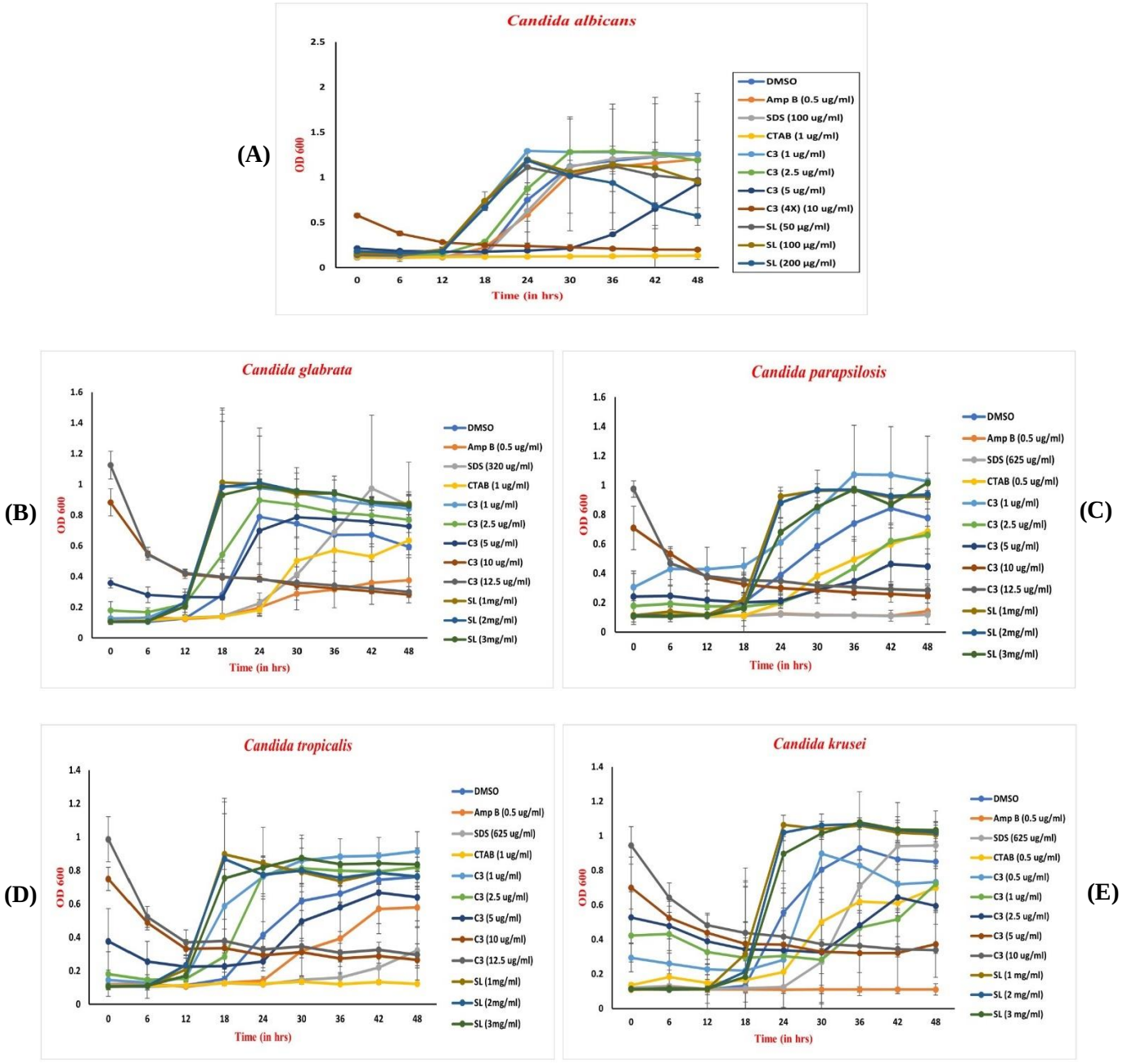



**Figure 11:** Time kill curve assay to determine the growth kinetics of *Candida* species in presence of drugs. IMT-C3 exhibited a fungicidal mode of killing above its MIC concentration while SL has a fungistatic mode of inhibition at higher concentrations only. DMSO was added in negative control and Amp B was used as positive control. Higher OD is observed at zero hour (in higher concentrations of IMT-C3 and SL) due to the cloudy effect generated by the addition of biosurfactants to the aqueous solution.

# CHAPTER 6: CONCLUSION AND DISCUSSION

Sophorolipid is an important precursor for various industrial processes such as personal care and beauty products, pharmaceutical products, environmental as well as bioremediation purposes, industrial and biotechnological processes, and many more. Biosurfactants, which includes sophorolipids, have demonstrated promising potential as sustainable substitute for synthetic surfactants. SL exhibits the potency for killing *Candida* cells either on its own or in combination of certain drugs as discussed in the performed experiments. Candidiasis which is caused by *Candida* species is the most prevalent form of nosocomial fungal infection. Immunocompromised patients are the most vulnerable to such infection. Since, SL has proven to show therapeutic effects with high efficacy and less toxicity, it becomes crucial for the present scenario in medical domain as well as anti-fungal drug pipeline. Even though sophorolipids, show significant potential, more research and development are needed to improve yield, lower costs, and optimise production processes. Realising the true capability of biosurfactants along with ensuring their widespread use across several domains in the future will depend on overcoming their limitations. In our study we have explored the potential of SL and its derivative IMT-C3 against various *Candida* species reported as most predominant fungal pathogens globally. We have investigated the plausible mode of anti-fungal inhibition mediated by the SL and its derivative, wherein we have observed that the derivative is more potent than the mother molecule. Also, IMT-C3 and SL can inhibit *Candida* species more efficiently than the conventional chemical surfactants which indicates that the inhibitory action is not only due its biosurfactant activity but a result of severe physiological stress to the yeast cells. Moreover, we show that the mother compound SL, could only inhibit the yeast pathogen at higher concentrations and takes time to act, on the other hand, the derivative designed to be more efficient, is able to kill the yeast cells within 6 hours of treatment. In addition, yeast cells can recover from the inhibitory effect of SL, while they fail to grow back in presence of IMT-C3, which indicates complete absence of viable cells or persistors in the condition. Thus, our study provides preliminary evidence to the potential of SL and IMT-C3 as potent anti-fungal agents.

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# Biocatalytic reduction of prochiral ketones to enantiopure alcohols by novel yeast isolates from unique biodiversity

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ABSTRACT

Enantiopure alcohols are the key building blocks required for the synthesis of a variety of pharmaceuticals, agrochemicals, flavour, aroma compounds and other fine chemicals. Preparation of these synthons using biocatalysis (enzyme/whole cells) is attaining high priority due to several benefits such as high enantio-, regio- and stereoselectivity, environment friendliness as well as better economic yields compared to their conventional chemical counterparts. In the present study, we report the synthesis of chiral naphthyl ethanols by whole cell-mediated biocatalysis of corresponding ketones. The yeast strains used as biocatalysts were isolates from the high altitude of the Himalayan mountain range. The strains showing potential as biocatalysts were found to be CHF–15P and CHF-15R which were further characterized by molecular analysis (ITS and D1/D2) and identified as *Rhodotorula kratochvilovae* (CHF–15P) and *Metschnikowia korensis* (CHF-15R), respectively. However, out of these two strains, CHF–15P (*Rhodotorula kratochvilovae*) exhibited better efficiency in converting the substrate 6′-methoxy-2′-acetonaphthone (conversion >95%; enantiomeric excess >99%). Further, the influence of various physicochemical parameters was studied to obtain the best condition for biocatalysis by both CHF–15P and CHF-15R cells. Finally, the best strain (CHF–15P) which was chosen for further studies exhibited excellent production of various naphthyl ethanols with >99% enantiomeric excess.

## 1. Introduction

In the past few decades, biocatalysis has gained enormous attention as environmentally benign greener technology for the production of molecules of industrial interest (Pollard and Woodley, 2007; Sun et al., 2017). Biocatalysis offers pollution-free greener routes for the chemo-, regio-, and enantioselective synthesis of various synthons and exploits the ability of the enzymes to catalyze the conversion of natural and non-natural substrates (Prier and Arnold, 2015; Wilson et al., 2015).

Enantiomerically pure alcohols are one of the most important intermediates for the synthesis of pharmaceuticals, agrochemicals, flavour, aroma and other fine chemicals (Ni and Xu, 2012). By employing biocatalysis (whole cell or isolated enzyme), prochiral ketones are asymmetrically reduced to produce optically active chiral secondary alcohols (Ni and Xu, 2012). Carbonyl reductases belonging to the oxidoreductase class of enzymes, are the key players carrying out such catalysis (Calvin et al., 2012). These catalysts require co-factors for carrying out the reactions. Since the reducing equivalents are too expensive, the experimental choice with purified enzymes needs extra supplementation of co-factors putting the burden on bioeconomy. The co-factor regeneration system has been introduced and established in many enzyme-catalyzed reaction systems (Calvin et al., 2012; Srivastava et al., 2015). However, the biocatalysis with whole cells remains the first choice due to their ability to efficiently regenerate necessary co-factors (Dascier et al., 2014). Moreover, whole cells provide a suitable environment to prevent conformational change of the enzyme (protein) structure in a non-conventional medium (de Carvalho, 2017). As a consequence, many whole-cell mediated biocatalytic systems have been

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